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The canine mast cell activation via CRP

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Abstract

We report here canine mastocytoma-derived cell (CMMC) activation via two pentraxin, limulus- and human-CRP. Mast cell chemotaxis was measured by Boyden's blindwell chamber. To confirm that the cell migration was chemotactic, "checkerboard" analysis was performed. We used Fura-2 to investigate CRP-mediated cytosolic calcium elevation. To examine whether CRP-induced stimulation is mediated through G-proteins, CMMC were incubated with pertussis toxin (PTx) before use in chemotaxis assay and Ca²⁺ mobilization. CMMC migration in response to CRP was both chemokinetic and chemotactic. Limulus-CRP induced a transient Ca²⁺-mobilization dose-dependently. Preincubation of the cells with PTx inhibited CRP chemotaxis and Ca²⁺-mobilization, suggesting that G-proteins of the Gi-class are involved in the chemotaxis. We suggest that CRP may participate in the migration of mast cells to inflamed tissues during an acute-phase response. CRP-mediated recruitment of mast cells might play an important role in hypersensitivity and inflammatory processes.

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Mast cell accumulation occurs in certain inflammatory conditions that include host response to microbes, perioperation tissue repair and fibrosis, delayed hypersensitivity reactions, neoplasia, allergy, and in chronic conditions such as inflammatory bowel disease, rheumatoid arthritis, and asthma [1]. Mature mast cells are resident cells found in most tissues. For a long time, they have been considered as stationary cells with no property to migrate [1,2]. However, the factors that control migration of mast cells to sites of inflammation and tissue repair remain largely undefined. Directed migration (chemotaxis) of mast cells within tissue may be the most speedy and reasonable mechanism, which increases the number of mast cells. The use of chemotaxis assay has allowed the identification of several mast cell chemoattractants. In murine mast cells, such as laminin

[3], interleukin-3 (IL-3)[4], stem cell factor (SCF) [5], transforming growth factor β (TGF β) [6], and monocyte chemoattractant protein-1 (MCP-1) [7] were found to promote mast cell migration. In contrast, SCF [8] and anaphylatoxins (C3a and C5a) [9] have been reported to induce chemotaxis in human mast cells.

Some of the pathological conditions that induce mast cell hyperplasia are also associated with high serum concentrations of acute-phase protein such as CRP. Serum concentrations of CRP in humans increase from less than one to hundreds of mg/ml as part of the inflammatory response to perioperation period, infection or injury [10]. It has been said that CRP was synthesized only in the liver, but CRP is expressed in macrophages [11]. Recent reports have indicated that CRP is not just a marker of underlying inflammation, but also is directly involved in the pathogenesis of arteriosclerotic lesions [12,13], the tissue damage that accompanies acute myocardial infarction [14], and the trigger of attack of intrinsic infectious type asthma.

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We have already demonstrated that IgG-mediated histamine release from canine cutaneous mastocytoma-derived cells (CM-MC) [15] and have investigated the expression of Fc γ receptors on the cell, monomeric IgG-binding to the cell, and IgG-mediated or IgE-mediated signal transduction in CM-MC [16]. In the present study, we used a modified Boyden's blindwell chamber chemotaxis assay to evaluate the potency of CRP as a chemoattractant for CMMC. We propose CRP as an additional group of chemotactic factors through which CMMC is recruited to sites of inflammation.

Materials and methods

Cells and buffers. The CMMC derived from canine cutaneous mastocytoma were passaged in the RPMI 1640 medium with 10% heat-inactivated FCS. The Pipes-ACM buffer used for the experiments consisted of 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, 0.1% BSA, and 10 mM Pipes (pH 7.4). Human-CRP were from (Chemicon, Temecula, Canada); Limulus-CRP were from (Sigma, St. Louis, USA). All concentration of CRP was nontoxic for CMMC as assessed by trypan blue.

CMMC chemotaxis checkerboard analysis. CMMC chemotaxis was measured by Boyden's blindwell chamber technique using a 48-well multi-well chamber and an 8- μ m-pore-size membrane (pvp free) (Neuroprobe, Gaithersburg, MD). Cell migration was expressed as the average of five randomly selected high power fields per well. To confirm that the cell migration was chemotactic, "checkerboard" analysis was performed. Each combination was tested in triplicate. The location of the various combinations in the 48-well manifold was randomized. The length of time required for CMMC chemotaxis to CRP was measured by varying the incubation time of the chemotaxis chambers. A total of 100 μ g/ml of CRP was used to examine whether CRP-induced stimulation is enhanced by IgG cross linking. CMMC were activated, in some experiments, by the addition of goat anti-canine IgG (10 μ g/ml, Bethyl, Montgomery, TX, USA) in upper wells after sensi-

tized with canine IgG (10 μ g/ml, ICN Pharmaceuticals, Aurora, OH, USA) for 30 min at 37 °C. The chemotaxis inhibition was measured by using various concentrations (10–200 μ g/ml) of goat anti-human-CRP (Bethyl, Montgomery, TX, USA) before human-CRP stimulation. Various concentrations of goat anti-human-CRP were added to 100 μ g/ml human-CRP for 30 min at 37 °C before chemotaxis. CMMC were incubated with 10, 30, and 100 ng/ml of PTx (Sigma Chemical, St. Louis, MO) in FCS complete medium for 120 min at 37 °C. The cells were then washed twice and resuspended in complete medium before use in chemotaxis assay.

Measurement of the intracellular calcium mobilization and histamine release. [Ca²⁺]_i was calculated as described in a previous paper [17]. Ca²⁺ mobilization and preincubation with 10, 30, and 100 ng/ml of PTx was also measured. Histamine were measured using *o*-phthalaldehyde fluorometric technique modified for autoanalysis as described [18].

Results and discussion

Chemotaxis response of CMMC to limulus- and human-CRP

The chemotactic activity of CRP for canine mast cell was determined in a modified Boyden's chamber. As shown in Table 1 (A: limulus and B: human), chemokinesis was present, since even when no gradient existed, the number of cells that migrated increased as the concentration of CRP increased. The number of migrated cells per hpf was 13.3 \pm 0.6 (with limulus-CRP), 11.0 \pm 1.0 (with human-CRP) and 0.7 \pm 0.6 (without stimulation), respectively. Cell migration was measured in various concentrations of CRP, both in the presence and absence of gradients of the chemoattractant. The cell migration was chemotactic, since migrated cells increased in the presence of gradient. Thus, CMMC migration in

Table 1
"Checkerboard" analysis of cell migration

	Concentration ^a above membrane ^b			
	0	50	100	200
Concentration ^a below membrane ^c				
(A) <i>Limulus-CRP</i>				
0	0.7 \pm 0.6	0.3 \pm 0.6	0.3 \pm 0.6	0.7 \pm 0.6
50	5.3 \pm 0.6	2.3 \pm 1.0	2.0 \pm 1.0	1.3 \pm 0.6
100	13.3 \pm 0.6	6.7 \pm 0.6	5.0 \pm 1.0	2.0 \pm 1.0
200	16.3 \pm 0.6	8.7 \pm 0.6	3.7 \pm 0.6	2.7 \pm 0.6
(B) <i>Human-CRP</i>				
0	1.0 \pm 0.0	0.7 \pm 0.6	0.3 \pm 0.6	0.3 \pm 0.6
50	3.3 \pm 0.6	1.7 \pm 0.6	0.3 \pm 0.6	0.7 \pm 0.6
100	11.0 \pm 1.0	3.3 \pm 0.6	2.0 \pm 0.0	1.0 \pm 1.0
200	15.3 \pm 2.1	8.7 \pm 0.6	2.7 \pm 0.6	2.3 \pm 0.6

In the top wells of the chemotactic chamber, various concentrations of CRP (limulus and human) (0, 50, 100, and 200 μ g/ml) were applied together with the target cells. In the bottom wells, the same concentrations of CRP were placed such that all possible combinations above and below the filter were tested.

^a Concentration of CRP respectively in medium (μ g/ml).

^b CM-MC cell suspension at 1.5×10^6 cells/ml in medium.

^c Concentration of CRP respectively as a chemoattractant. Number of migrated cells is indicated as average in triplicate \pm SEM.

response to CRP was both chemokinetic and chemotactic. To examine the effect of extracellular Ca^{2+} concentration, we used Ca^{2+} free solution (Pipes EGTA) and Pipes ACM ($\text{Ca}^{2+} = 3\text{mM}$) instead of Pipes ACM ($\text{Ca}^{2+} = 1\text{mM}$). The number of migrated cells per hpf was no different (data not shown). Chemotaxis to CRP was independent of extracellular Ca^{2+} concentration.

Figs. 1A (limulus) and B (human) show a typical bell-shaped dose response curve of mast cell migration with higher concentrations resulting in the loss of directed migration. However, in contrast to other mast cell chemoattractants such as SCF and C3a [8,9], CRP induced mast cell migration in a more narrow concen-

tration range. The optimal concentration of CRP for the induction of mast cell migration was $250\text{ }\mu\text{g/ml}$, which is higher than the normal serum level ($<1\text{ }\mu\text{g/ml}$), but well below the levels seen in inflammatory conditions. Figs. 1C (limulus) and D (human) show that the number of CMMC increased with time and reached a plateau at 2 h. As mast cell chemotaxis to CRP is enhanced by mast cell activation, we also determined if activation by IgG-dependant stimuli would enhance their chemotactic responsiveness. Figs. 1E (limulus) and F (human) show that CMMC chemotaxis to CRP is mildly enhanced after activation of CMMC with anti-IgG after sensitization of the cells with IgG.

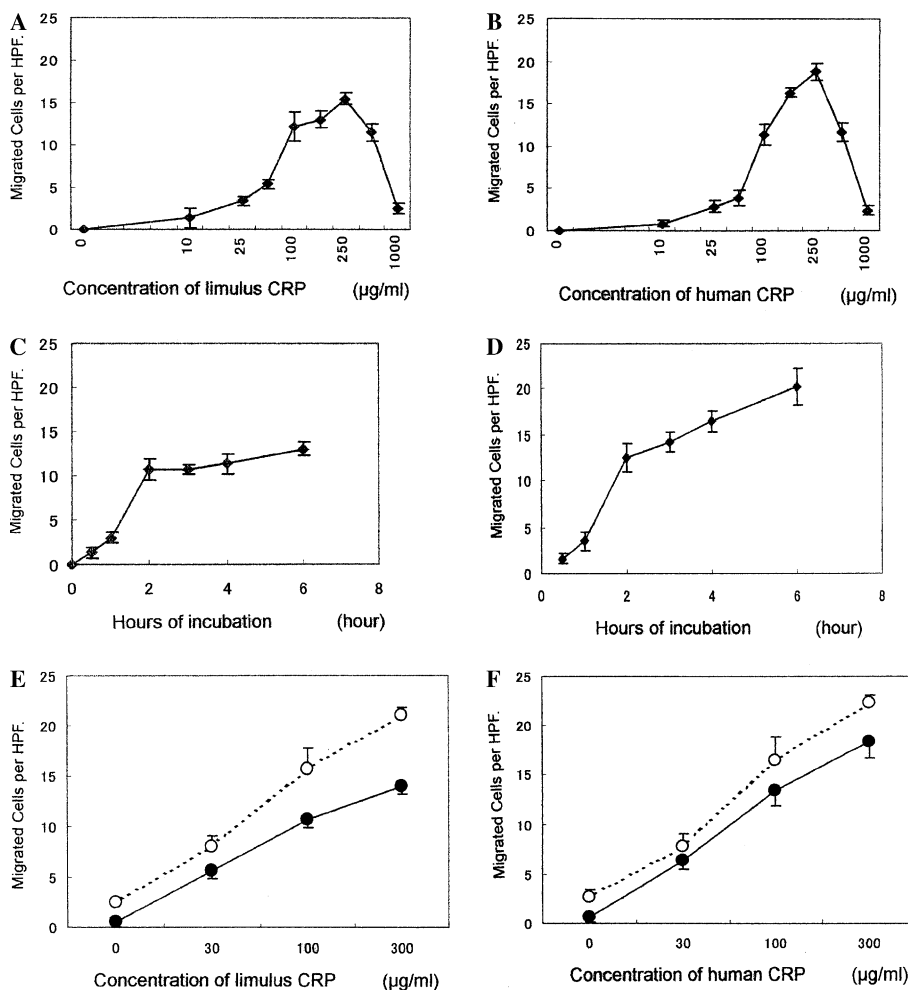


Fig. 1. Limulus-CRP was placed into each of the bottom wells; above the filter CMMC prepared as described were placed into each of the top wells. The Chambers were then incubated at 37°C , $5\% \text{CO}_2$ for 2 h. After incubation, cells on the top of the filter were removed by scraping. The filter was then stained with Diff Quick stain. CMMC chemotactic activity was determined as the total number of migrated CMMC counted in 10 hpf (high power field) average using a light microscope (BX51, Olympus, Osaka, Japan) at $400\times$ magnification. (A) (limulus) (B) (human) Dose-dependent chemotaxis of CMMC to CRP. Vertical axis: CMMC chemotaxis expressed as the number of cells migrated of per hpf. Horizontal axis: concentration of CRP. Bar represents the SEM for three individual experiments. (C) (limulus) (D) (human) Time dependence chemotaxis of CMMC to CRP ($100\text{ }\mu\text{g/ml}$) CMMC demonstrated chemotactic migration in response to $100\text{ }\mu\text{g/ml}$ of CRP. Vertical axis: CMMC chemotaxis expressed as the number of cells migrated per hpf. Horizontal axis: incubation time of chemotaxis chamber. Bar represents the SEM for three individual experiments. (E) (limulus) (F) (human) CMMC were activated by the addition of canine anti-IgG after sensitized with canine IgG. Migration of IgG activated CMMC cell to limulus-CRP. Open circles represent the inclusion of anti-IgG ($10\text{ }\mu\text{g/ml}$) after sensitized with IgG ($10\text{ }\mu\text{g/ml}$). Bar represents the SEM for three individual experiments.

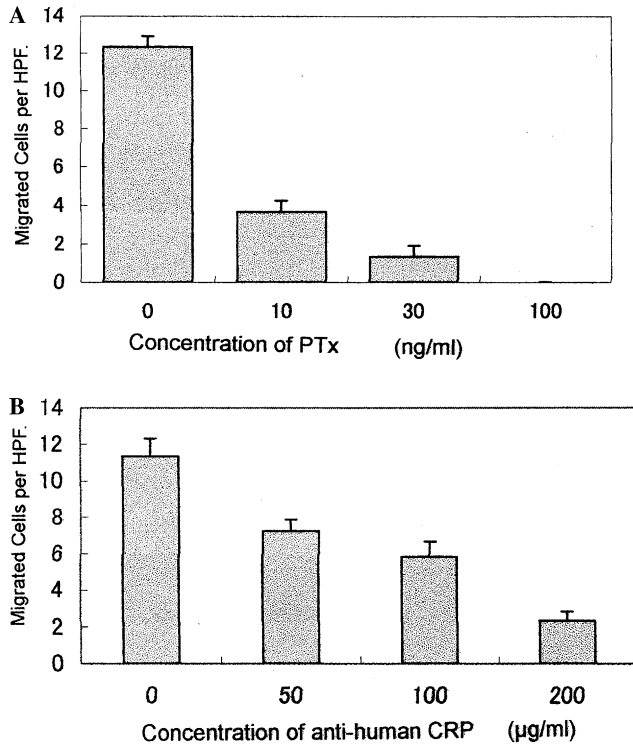


Fig. 2. (A) The chemotaxis inhibition was measured by using various concentrations (50 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$) of goat anti-human-CRP, before human-CRP stimulation. Various concentrations of goat anti-human-CRP were added to 100 $\mu\text{g/ml}$ human-CRP for 30 min at 37 $^{\circ}\text{C}$ before chemotaxis. Pretreatment with goat anti-human-CRP (50–200 $\mu\text{g/ml}$) partially inhibited the migratory response to limulus-CRP (100 $\mu\text{g/ml}$) which decreased in a dose-dependent manner. (B) CMMC were incubated with PTx before use in the chemotaxis assay. Limulus-CRP stimulates the migration of CMMC via a PTx-sensitive G protein. Pretreatment with PTx (10 ng/ml) partially inhibited the migratory response to limulus-CRP (100 $\mu\text{g/ml}$) (71% inhibition, $P = 0.05$) and PTx (100 ng/ml) totally inhibited (100% inhibition).

To examine whether CRP-induced stimulation is mediated through guanine nucleotide-binding proteins (G proteins), CMMC were incubated with pertussis toxin before use in the chemotaxis assay. Pretreatment with PTx (10 ng/ml) partially inhibited the migratory response to limulus-CRP (100 $\mu\text{g/ml}$) and PTx (100 ng/ml) totally inhibited (Fig. 2A). Thus, chemotactic response of mast cells to CRP is pertussis toxin sensitive. As shown in Fig. 2B, pretreatment with goat anti-human-CRP (50–200 $\mu\text{g/ml}$) partially abrogated the migratory response to 100 $\mu\text{g/ml}$ of limulus-CRP in a dose-dependent manner.

Measurement of the intracellular calcium mobilization and histamine release

In various cell types, migration was found to be associated with alteration in $[\text{Ca}^{2+}]_i$, as an early step in signal transduction [19]. Therefore, $[\text{Ca}^{2+}]_i$ changes in CMMC in response to CRP were measured. CRP-

mediated intracellular calcium mobilization was examined using the calcium indicator Fura-2-AM as shown in (Figs. 3A and B). The $[\text{Ca}^{2+}]_i$ increase by CRP stimulation occurred and the rapid increase in the early interval merged into a very slow increment. Limulus-CRP (100 and 300 $\mu\text{g/ml}$) induced a rapid and transient Ca^{2+} mobilization dose-dependently. In contrast, no significant elevation of $[\text{Ca}^{2+}]_i$ was observed in resting cells (data not shown). Ca^{2+} mobilization of CMMC in response to CRP was different from anti-IgG activation (Fig. 3C). To examine whether CRP-induced stimulation is mediated through guanine nucleotide-binding

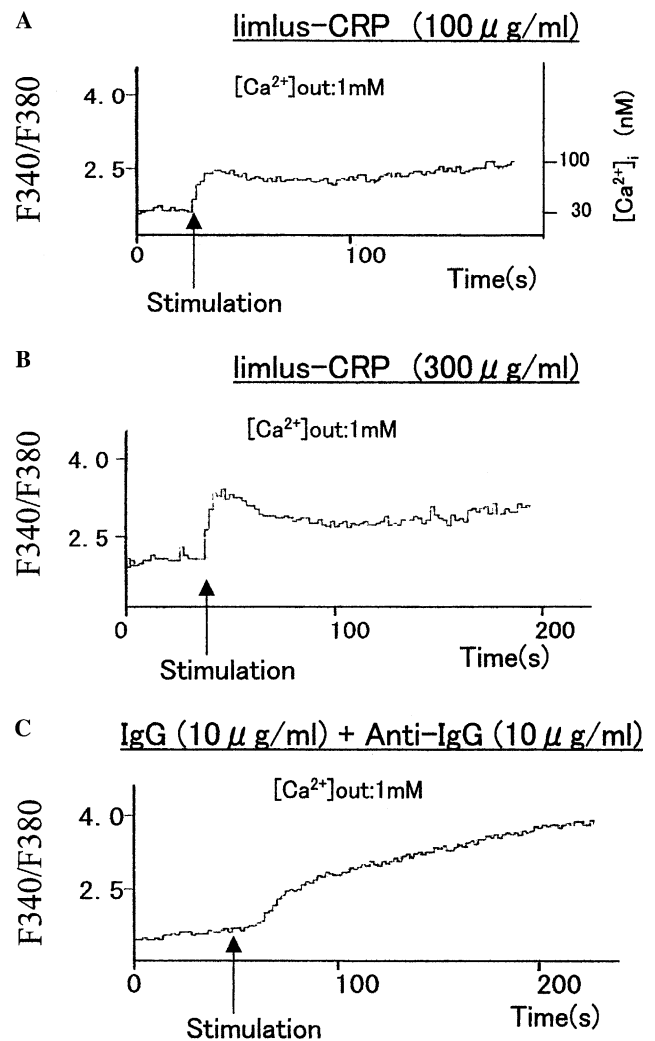


Fig. 3. CMMC (5×10^5 cells/ml) were resuspended in 1.5 ml of the buffer and were stimulated with limulus-CRP (30, 100, and 300 $\mu\text{g/ml}$) for 30 min at 37 $^{\circ}\text{C}$. The cells were loaded with Fura-2-AM (6 μM , Dojindo, Kumamoto, Japan) for the last 15 min of the stimulation. During the measurement of $[\text{Ca}^{2+}]_i$, cells were stirred at 37 $^{\circ}\text{C}$ and illuminated alternately with two excitation wavelengths (340 and 380 nm) and fluorescence at 495 nm was measured with a Shimadzu RF-5000 spectrophotometer (Kyoto, Japan). Stimulated with limulus-CRP 100 $\mu\text{g/ml}$ (A), stimulated with limulus-CRP 300 $\mu\text{g/ml}$ (B) a rapid $[\text{Ca}^{2+}]_i$ increase was observed in the CM-MC cells sensitized with 10 $\mu\text{g/ml}$ of canine IgG and stimulated with 10 $\mu\text{g/ml}$ of anti-canine IgG. (Positive control) (C).

proteins (G-proteins), CMMC cells were incubated with pertussis toxin before use in measurement of the intracellular calcium mobilization. The $[Ca^{2+}]_i$ elevation response to limulus-CRP was partially abolished after pretreatment with PTx (10 ng/ml) and was totally abolished by PTx (100 ng/ml) (Figs. 4A–D).

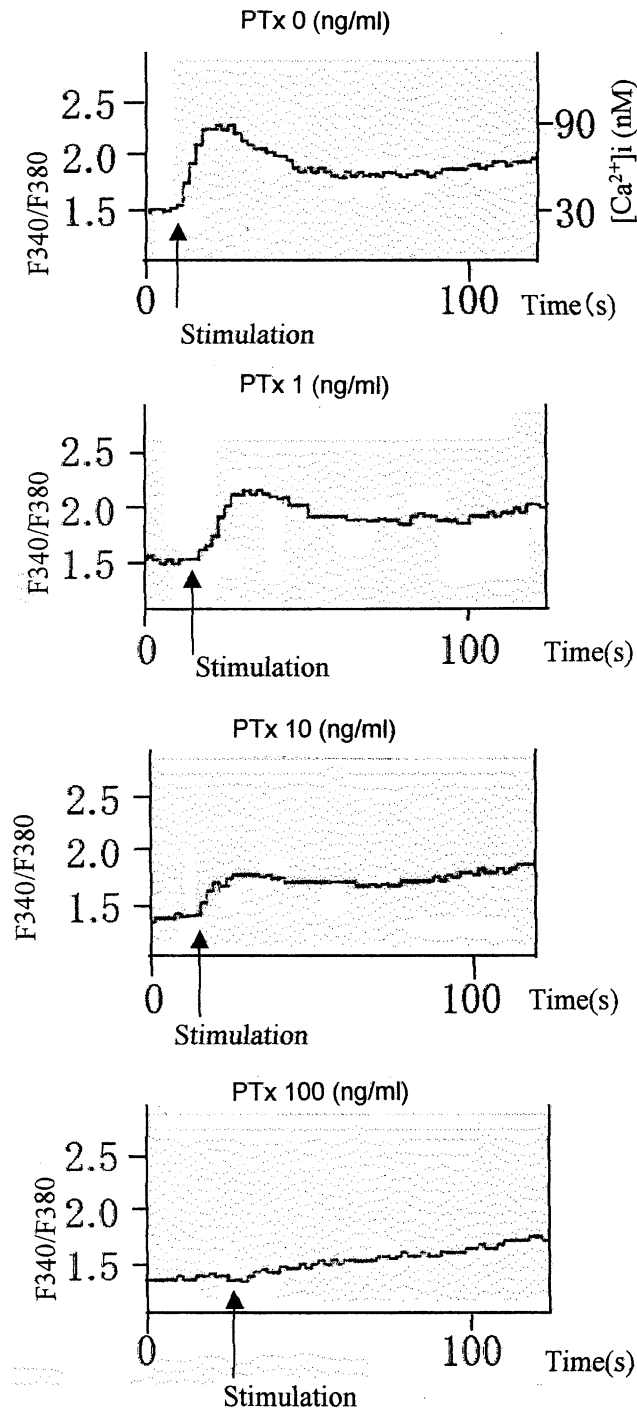


Fig. 4. After treatment of CMMC with PTx at (0.1.10.100) ng/ml for 120 min, cells were stimulated with limulus-CRP (300 μ g/ml) Pre-treatment with PTx (100 ng/ml) totally inhibited (100% inhibition) the $[Ca^{2+}]_i$ elevation response to limulus-CRP.

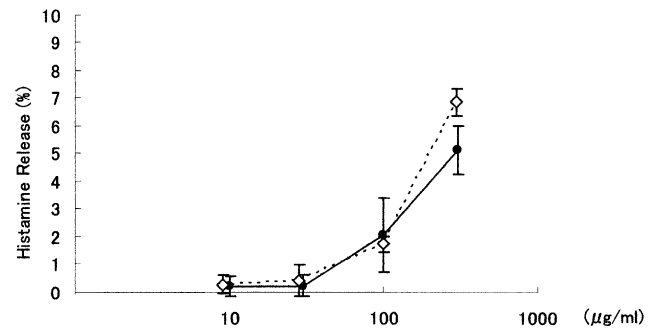


Fig. 5. Histamine release from CMMC induced by limulus-CRP (solid line) and human-CRP (broken line). Cells were incubated with the stimuli at 3 °C for 30 min. Bar represents the SEM for three individual experiments.

Limulus- and human-CRP induced histamine release. The maximum histamine release from CMMC was $5.1 \pm 0.9\%$ at 300 μ g/ml of limulus-CRP and $6.9 \pm 0.5\%$ at 300 μ g/ml of human-CRP (Fig. 5). RBL-2H3 (rat basophilic leukemic cell-2H3) and human basophils did not release histamine by limulus- and human-CRP.

The main biologic function of CRP lies in its ability to recognize pathogens and damaged cells of the host and to mediate their elimination by recruiting the complement system and phagocytic cells. Functions of CRP attributable to direct interactions with phagocytic cells have been known for many years [20], but it was only recently shown that CRP binds to the high affinity IgG receptor, Fc γ RI, on mononuclear cells and on COS cells transfected with a cDNA encoding this receptor [21,22]. In our study, CM-MC cell chemotaxis was enhanced after activation of CMMC with anti-IgG after sensitization of the cells with IgG (Fig. 3C). In addition, pretreatment with PTx inhibited CRP-mediated chemotaxis of CMMC (Fig. 4) and the Ca^{2+} mobilization, indicating that guanine nucleotide-binding proteins (Gi proteins) are involved in signal transduction pathways in CMMC. These results indicate that mast cell chemotaxis is mediated by distinct signal transducing pathways from an immuno-receptor mediated one.

We used two members of the pentraxin family, limulus- and human-CRP. The cross-reactivity of limulus-CRP with the human one existed due to antigenic similarity between limulus- and human-CRP [23]. Limulus-CRP shares many of the biological functions of immunoglobulin such as complement fixation and phagocytes [24]. The coding regions of the limulus-CRP and human-CRP genes share approximately 30% identity and two stretches of highly conserved regions, one of which falls in the region proposed as the phosphorylcholine binding site, while the other site is very similar to the consensus sequence required for calcium binding in calmodulin and related protein [24,25]. In conclusion, we propose that CRP is one of the G protein-mediated chemoattractants which governs mast cell distribution and promotes their accumulation in acute inflammatory phase.

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